Molecular Ecology Notes (2007) 7, 1375–1378 doi: 10.1111/j.1471-8286.2007.01892.x

PRIMER NOTE

A multiplex set of microsatellite markers for the scarlet rosefinch (*Carpodacus erythrinus*)

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Abstract

Cardueline finches have become important models in studies of sexual selection and evolution of carotenoid-based ornamentation. Here, we describe eight new polymorphic microsatellites isolated from the Scarlet rosefinch (*Carpodacus erythrinus*) and four from the House finch (*Carpodacus mexicanus*). Together with the cross-species amplification of additional loci, originally published for two species of songbirds, we optimized a multiplex panel for *C. erythrinus* allowing genotyping of 22 polymorphic loci. Number of alleles and heterozygosity per locus in a sample of 34 individuals ranged from three to 38 and from 0.27 to 0.94, respectively.

Keywords: *Carpodacus*, cross-amplification, Fringillidae, heterozygosity, microsatellites

Received 22 April 2007; revision accepted 4 June 2007

Finches of the genus *Carpodacus* have become important models in studies of sexual signalling (Hill 2002). Molecular techniques to determinate parentage, heterozygosity and genetic complementarity are important for understanding mechanisms of sexual selection in these birds (Oh & Badyaev 2006; Albrecht *et al*. 2007). Here, we report on a set of polymorphic microsatellites for the Scarlet rosefinch *Carpodacus erythrinus*, developed by screening of genomic libraries of two *Carpodacus* finches and by cross-species amplification of loci from related species.

Enrichment technique following Estoup & Martin (1996) was used to develop microsatellite markers for *C. erythrinus*. Genomic DNA was extracted from ethanol-preserved blood and digested using *Rsa*I restriction enzyme (Promega). A set of 500–900-bp fragments was selected, purified and ligated to *Mlu*I oligo adaptors (RSA21 and phosphorylated RSA25 from Edwards *et al*. 1996). Biotinylated oligo probes [$(TC)_{10}$ and $(TG)_{10}$] were hybridized to the ligated DNA and selected using streptavidin magnetic particles (Promega). Polymerase chain reactions (PCR) were performed on the microsatellite-enriched eluate using one of the oligo adaptors (RSA21) as a primer. The amplification products were purified and ligated into a plasmid vector (Promega), transformed into JM109 competent cells (Promega), and plated onto Luria-Bertani (LB) agar medium. Positively transformed cells were grown on LB agar, transferred onto Hybond-N + membranes (Amersham) and screened using digoxigenin-end-labelled $(TC)_{10}$ and $(TG)_{10}$ probes. Clones containing repetitive DNA were sequenced and PCR primers were designed from unique nucleotide sequence regions flanking microsatellites using NETPRIMER (Premier Biosoft International). PCR conditions were optimized to produce clear bands on agarose gels, and for 10 loci (Table 1), forward primers were labelled with a fluorescent dye (Applied Biosystems).

Four additional polymorphic loci (Table 1) were identified from CTTT- and CA-enriched genomic DNA libraries for *Carpodacus mexicanus* (Hamilton *et al*. 1999; for detailed protocols see www.uga.edu/srel/). Shortly, genomic DNA was digested with *Rsa*I (New England Biolabs) and

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Table 1 Characterization of 22 microsatellite loci for 34 genotyped Scarlet rosefinches

Locus	GenBank Accession no.	Repeat type	Primer sequence (5′–3′)	Multiplex PCRs			Size range (bp)
				Set	μM	Dye	
CE207*	EF197158	$(CA)_9$	F: AGTCCAGGAGAGTTGAGCAT R: CAGTGTGGTTCAACAGCTCC	c	0.20	FAM	218–273
CE165*	EF197159	$(CA)_{12}CG(CA)_4$	F: GTTATCGCCAGCAACACAAC R: TAAGCAGCAGCAGTAGTTCG	b	0.25	VIC	96–130
CE 31*	EF197160	$(GA)_9$ GG(GA)	F: AGGAGAACTTGCAGTAGAGG R: AGTCTCGCTGGCACATCT	b	0.50	PET	152–171
CE150*	EF197161	$(GA)_8$	F: CCTTCAATCACTCCTTCC R: GCACCTGCAGCTGTAAAT	d	0.20	FAM	163–177
CETC215*	EF197163	$(TC)_{12}$	F: ACCTGACCTAGCTGATGTTC R: GCTGCCTGGCAGTAATAA	b	0.15	VIC	161–189
CE152*	EF197162	$(CA)_4$ CG$(CA)_7$	F: TAAGACTGGAAGGCAGCA R: GCCAACAGCGTTCAGATC	a	0.15	HEX	131–182
CE67*	EF560648	$(TG)_{18}$	F: TGTTTCCTTGGCCTGTCA R: CACTCACTCTGCACTCAACT	—	0.3	PET	112–161
CE147*	EF560649	$(TG)_{16}$TT $(TG)_8$	F: CAGTAGTTGGAGTAGGAAGG R: CAGGAGCTGGATGGTTGTTA	—	0.5	NED	297–302
CM026†	EF495348	$(TG)_8$	F: ATACTGTGGGTGTGGGGTTT R: CCTGCTGGCACACTTTGTAG	a	0.10	HEX	184–186
CM014†	EF495347	$(TG)_7$	F: GGAGGAAGGAAACTGTTTTGC R: CCCATGGCACATGTTGACTA	d	0.10	NED	154–188
CM001E†	EF495349	$(GAAA)_{16}GAT(GATA)_{11}$	F: TATAAGGTCCTCACCGAGCC R: CACTGCATCTTTCCAAAGCTAA	d	0.30	NED	199–291
CM008E†	EF495350	$(GAAA)_{13}$	F: CATGATCCATGTCAACAG R:AGTAGTCTGACAGGTCC	a	0.50	HEX	334–423
Hofi 17§	AY843216	$(TTAG)_{10}$TTT GTATG$(TTTG)_9$	F: GCATACACAGCACGCCAAACACAAAG R: CATGTGCTGTGTTGGGTGAGTCTGTGG	a	0.25	NED	156–181
Hofi 52§	AY843220	$(TAGG)_7TATG(TAGA)_{13}$	F: GCGGGAATTCCAGACAAACT R: CACTGAACTATGCTGACACTATGA	a	0.10	HEX	233–292
Hofi 23§	AY843218	$(GT)_{11}$	F: TTCCCACTGTTTGTAAATAAGA R: CAGTGGCAGTTGCTATGAG	b	0.15	FAM	130–136
Hofi 3§	AY843213	$(CA)_2GA(CA)_{15}$	F: CTTAAATTTACATCCTGCCAACC R: AGTCAGGGATAGGGAGAAGAAGTG	b	0.10	NED	133–148
Hofi 24§	AY843219	$(GT)_{10}$	F: CTTCAGCCCTTTGCACAGGCAGTTTG R: GAGAGCCAACAAACACCCGTCAGTGG	c	0.10	NED	119–122
Hofi 7§	AY843215	$(AC)_5AT(AC)_{28}$	F: AGTACCATCCTTCTCTGTCTGC R: ATTCTTGCTCCTATTTTATTCTCC	d	0.20	FAM	234–260
Hofi 5§	AY843214	$(TG)_{12}$	F: ACTGCAGAAAGCGATCACATTACG R: GGCCCTCATTCCTCCTGCTTAC	d	0.25	HEX	349–387
LOX 1‡	Y16820	$(CTTT)_{30}$	F: ATGATGGTAAGTCTAATGAAAGC R: CCACACACATTCACTCTATTG	c	0.50	NED	272–298
LOX 3‡	Y16822	$(CTTT)_{21}$	F: TTCTGTGGTGAAGTTTTCTGGAG R: CCAACCCATTCCATGACAAC	c	0.20	FAM	90–98
LOX 6‡	Y16824	$(CTTT)_{19}$	F: ACAAATAACATAGGTCAGAAGC R: GCTCTATAACTTTGTGATTTTGC	d	0.20	HEX	158–363

Locus, **Carpodacuserythrinus* (developed by the TA group); †*Carpodacus mexicanus* (developed by the GEH group); §*C. mexicanus* (Hawley 2005); ‡*Loxia scotica* (Piertney *et al*. 1998). Set, number of multiplex PCR (loci CE67 and CE147 were amplified separately); μM, μM of each primer in multiplex PCRs; Dye, 5′ fluorescent label.

blunt-end ligated to the SNX adaptor (Hamilton *et al*. 1999). Following ligation, the adapter-ligated restriction fragments were PCR amplified using SNX-24 forward sequence as primers. PCR-amplified fragments were then subjected to selection using hybridization with biotinyated oligonucleotides [$(CTTT)_8$, Invitrogen] and the hybrids captured using streptavidin-coated paramagnetic beads (Dynabeads, Dynal Biotech Inc.). Selected fragments were then cloned (Invitrogen) and colonies were probed with $(CTTT)_8$ oligonucleotides labelled with P^{32}. Inserts from

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Table 2 Levels of variability at 22 polymorphic microsatellite loci in *Carpodacus erythrinus*

Locus	*n*	N_a	H_O	H_E	*P* value	Frequency of null alleles
CE207	34	13	0.88	0.90	0.429	0
CE165	34	15	0.82	0.83	0.205	0.0218
CE 31	34	10	0.59	0.85	**0.013**	0.1312
CE150	34	10	0.85	0.84	0.219	0.0094
CETC215	34	13	0.94	0.90	0.206	0
CE152	34	13	0.85	0.86	0.111	0
CE67	34	16	0.65	0.90	**0.002**	0.1291
CE147	34	4	0.62	0.65	0.881	0.0069
CM026	34	3	0.47	0.54	0.075	0.0078
CM014	34	3	0.41	0.45	0.360	0.0374
CM001	34	29	0.94	0.96	**0.018**	0.0473
CM008E	34	38	0.81	0.98	**< 0.001**	0.0764
Hofi 17	34	15	0.94	0.90	0.197	0
Hofi 52	32	20	0.88	0.94	0.053	0.0209
Hofi 23	34	4	0.27	0.60	**< 0.001**	0.2073
Hofi 3	34	9	0.35	0.73	**< 0.001**	0.2033
Hofi 24	34	4	0.59	0.56	**0.015**	0.0375
Hofi 7	34	13	0.32	0.87	**< 0.001**	0.2774
Hofi 5	30	19	0.90	0.93	**0.017**	0.0304
LOX 1	30	8	0.57	0.65	0.128	0.0278
LOX 3	34	3	0.35	0.39	0.227	0.0127
LOX 6	34	31	0.38	0.95	**< 0.001**	0.2654

n, number of individuals successfully genotyped per locus; N_a, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity. Loci that significantly deviated from Hardy–Weinberg equilibrium at $P < 0.05$ are in bold and frequency of null alleles was calculated by FREENA software.

positive colonies were then amplified and sequenced. An alternate CA-motif library was created in a similar manner; however, rather than selecting positive clones from a Southern blot before sequencing, approximately 100 randomly selected clones were amplified and sequenced. Primers were designed using NETPRIMER (Premier Biosoft International).

We also tested the cross-species amplification of previously published primers originally identified for *Loxia scotica* (Piertney *et al*. 1998) and *C. mexicanus* (Hawley 2005). Together with novel markers, we successfully amplified 22 polymorphic loci in *C. erythrinus* (Table 1). Multiplex PCRs were performed on a Mastercycler ep gradient S (Eppendorf) in a total volume of 10 μL using the QIAGEN Multiplex PCR Kit following the manufacturer's protocol, with an annealing temperature of 56 °C for 90 s. Loci CE67 and CE147 were amplified in 10-μL reaction containing 0.2 mM of each dNTPs, 1× *Taq* DNA buffer (Fermentas), 3 mM $MgCl_2$ (1.5 mM for CE67), and 0.5 U of *Taq* DNA polymerase (Fermentas) at the following temperatures: initial activation step at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s (64 °C for CE67), extension at 72 °C for 30 s and final extension at 72 °C for 10 min.

One microlitre of PCR product was mixed with 0.2 μL of GeneScan-500 LIZ or 500 ROX Size Standard (Applied Biosystems) and 12 μL of formamide (Applied Biosystems) and electrophoresed using ABI PRISM 3130 sequencer (Applied Biosystems). Allele sizes were estimated using GENEMAPPER 3.7 (Applied Biosystems). Expected and observed heterozygosities and the tests for genotypic linkage disequilibrium and deviations from Hardy–Weinberg equilibrium were calculated in the software GENEPOP version 3.3 (Raymond & Rousset 1995). The frequency of null alleles was calculated using FREENA (Chapuis & Estoup 2006).

To assess genetic variation, we genotyped 34 rosefinches from one locality (Vltava river valley, Šumava Mountains, Czech Republic). The number of alleles per locus ranged from three to 38 and expected heterozygosity values ranged from 0.39 to 0.98. Observed and expected heterozygosity values conformed to Hardy–Weinberg equilibrium ($P > 0.05$) for 12 out of 22 loci. The significant deficit of heterozygotes at eight loci may be caused by the presence of null alleles in the studied population (Table 2). After Bonferroni correction, there was indication of significant linkage disequilibrium for 12 pairs of loci including 13 different loci which is on the border of statistical error (5.2% of 230 compared pairs). Optimized microsatellite markers may be used to study the genetic mating system of rosefinches, including mating with respect to genetic complementarity and heterozygosity.

Acknowledgements

The work was supported by the Grant Agency of the CR, project no. 206/06/0851. We thank Jan Schnitzer, Pavel Munclinger, Michal Vinkler, Marta Promerová and Olga Ševčíková for help with bird sampling. Development of house finch libraries in the GEH laboratory by H.L.M. was supported by NSF grant DEB-0077804.

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